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Rifampin as a Selective Agent for Isolation of Oral Spirochetes

S. B. LESCHINE AND E. CANALE-PAROLA*

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Spirochetes indigenous to the healthy gingival crevice of the human mouth were isolated directly from colonies in agar medium containing rifampin as a selective agent.

Stanton and Canale-Parola (9) recently isolated morphologically diverse spirochetes from the bovine rumen by means of a procedure that involved the use of the antibiotic rifampin as a selective agent. A major step in this procedure consisted of preparing serial dilutions of rumen fluid in an agar medium containing rifampin. The rifampin present in the agar medium was inhibitory to the growth of many rumen bacteria other than spirochetes. Thus, the inhibitory effect of rifampin markedly increased the numerical ratio of spirochete colonies to other colonies and facilitated the direct isolation of spirochetes. This method has advantages over conventional isolation methods (2, 7, 8) that are based on the ability of spirochetes to migrate through agar media or to pass through membrane filters. Such conventional methods select for spirochetes which migrate most rapidly through agar media or for spirochetes slender enough to pass through the pores of membrane filters. By using the rifampin method, the size and migration restrictions imposed by conventional procedures are avoided, and it is possible to obtain discrete colonies of slow-growing spirochetes from which pure cultures can be isolated.

We found that not only rumen spirochetes, but also various host-associated and free-living spirochetes isolated by methods not involving rifampin are resistant to relatively high concentrations of the antibiotic (S. B. Leschine and E. Canale-Parola, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I20, p. 87). This indicated that rifampin resistance may be a general characteristic of spirochetes, or that it may be widespread among these bacteria. It also supported the suggestion (9) that rifampin could serve as a selective agent for the isolation of spirochetes from environments other than the rumen.

In view of these considerations, we used the rifampin procedure to attempt the isolation of spirochetes from the gingival crevice of the human mouth. Gingival crevice debris was collected by means of sterile toothpicks from healthy sites (never diagnosed as diseased during dental examinations and asymptomatic at the time of collection). Toothpicks with adhering debris were placed in tubes, each containing 3 ml of GM-1 broth medium in an N_2 atmosphere. Medium GM-1 was prepared as described previously (1) except that it included 1.7% (vol/vol) heat-inactivated normal rabbit serum (Flow Laboratories), and the sodium bicarbonate solution was filter sterilized before it was added as a supplement to the medium. Furthermore, the medium was prereduced (10). The tubes were sealed with neoprene rubber stoppers, gently shaken to disperse the gingival crevice debris, and incubated at room temperature for 2 h. The number of spirochetes observed microscopically in the suspension fluid increased during this time, presumably because they swam free from pieces of debris. The suspension was serially diluted into tubes of melted agar medium GM-1 (0.7% Difco Noble agar; 45°C) containing rifampin (Sigma) (2 μg/ml, final concentration). Rifampin was dissolved in the sodium bicarbonate solution before it was filter sterilized and added to the medium. During these steps, the medium

TABLE 1. *Effect of rifampin on the cultivation of spirochetes*[a]

Rifampin concn (μg/ml)	Viable counts ($\times 10^5$)[b]		Spirochetes[c] (% total)
	Total bacteria	Spirochetes	
0	102	2.0[d]	2
1	5.7	2.0	35
2	3.9	1.1	28
5	3.2	1.7	53
10	1.4	1.1	79

[a] Gingival crevice debris was suspended in 3 ml of GM-1 broth and then serially diluted in tubes of GM-1 agar medium containing the concentrations of rifampin indicated.

[b] Colony-forming units per ml of gingival crevice debris suspension.

[c] Spirochetes expressed as percent total bacteria that formed colonies in the presence of the rifampin concentration indicated.

[d] This value was an estimation because of the large number of nonspirochetal colonies that developed in the absence of rifampin.

TABLE 2. *Morphological types of spirochetes isolated from the human mouth*[a]

Representative strain	Figure[b]	Cell size (μm)	Periplasmic fibrils per cell[c]	Cell coiling
H42	1A	0.1–0.2 by 3–5	2	Regular, tight
CC32	1B, 2A	0.1–0.2 by 5–15	2	Regular, tight
C41	1C, 2B	0.15–0.25 by 4–8	4–6	Irregular
H32	1D, 2C	0.2–0.3 by 8–16	10–12	Loose, forms secondary coils (3)

[a] Strains H42, CC32, and C41 were grown in GM-1 broth, and strain H32 was grown in NOS broth. All strains were examined after 7 days of growth at 35°C.

[b] Numbers refer to figures in this article.

[c] Determined by electron microscopy of negatively stained cells (9).

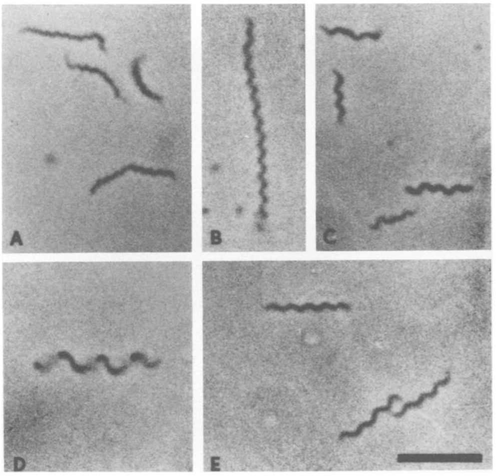

FIG. 1. *(A to D) Phase-contrast photomicrographs of representative strains of spirochetes isolated from the gingival crevice of the human mouth (wet mount preparations). (A) Strain H42; (B) strain CC32; (C) strain C41; (D) strain H32. Spirochetes were cultured as described in Table 2, footnote a. (E) Phase-contrast photomicrograph of a wet mount preparation of Treponema denticola strain 10 (4), included for comparison purposes. T. denticola was cultured in GM-1 broth for 7 days at 35°C. Photomicrographs were taken as described previously (9). All micrographs are at the same magnification. Bar = 5 μm.*

was maintained under a stream of N_2. The inoculated culture tubes were sealed with neoprene rubber stoppers and incubated at 35°C.

Spirochetes formed readily recognizable colonies (9) in the agar medium used. Typically, the spirochete colonies were spherical and increased in diameter as the spirochetes multiplied and migrated through the agar medium. Often they resembled cotton balls and were either dense or diffuse. Other spirochete colonies resembled nearly transparent bubbles with well-defined peripheries. Colonies or portions of colonies were removed from the agar medium with sterile Pasteur pipettes, and the cells were cloned by successive transfers in agar medium (serial dilutions, as described above).

Addition of rifampin markedly increased the ratio of the number of spirochete colonies to the number of total colonies (Table 1), thus facilitating both the enumeration and the isolation of spirochetes. The number of spirochetes present in gingival crevice debris varied from sample to sample; the sample used in the experiment outlined in Table 1 contained a higher than average proportion of spirochetes.

By means of the rifampin procedure, we isolated numerous strains of oral spirochetes representing several morphological types that differed in cell size, cell coiling pattern, and number of periplasmic fibrils (3) per cell (Table 2 and Fig. 1 and 2). In GM-1 agar medium, strain H42 formed very small (<3 mm diameter) diffuse colonies, strain CC32 formed larger (>5 mm diameter) and nearly transparent colonies with slightly defined peripheries, and strain C41 formed very dense cotton ball-like colonies. Strain H32 was isolated by the same procedure as described above except that NOS agar medium (containing rifampin, 2 μg/ml, final concentration) was used instead of GM-1 agar medium. NOS agar medium was prepared in the same manner as GM-1 medium (1) but contained the following (in grams per 100 ml of distilled water): heart infusion broth (Difco), 1.25; Trypticase (BBL Microbiology Systems), 1.0; yeast extract (Difco), 0.25; sodium thioglycolate, 0.05; L-cysteine hydrochloride, 0.1; L-asparagine, 0.025; glucose, 0.2; Noble agar (Difco), 0.7. The medium was supplemented with the following (in milliliters per 100 ml): 0.2% (wt/vol) thiamine pyrophosphate, 0.3; volatile fatty acids (1), 0.2; 10% sodium bicarbonate, 2.0; and heat-inactivated normal rabbit serum, 2.0. All supplements were filter sterilized. In this medium, strain H32 formed very large (>10 mm diameter), fairly dense colonies.

Many morphologically distinct spirochetes are usually seen in gingival crevice debris, and yet few have been cultivated in vitro (6). Possibly growth in vitro of these organisms is impeded by

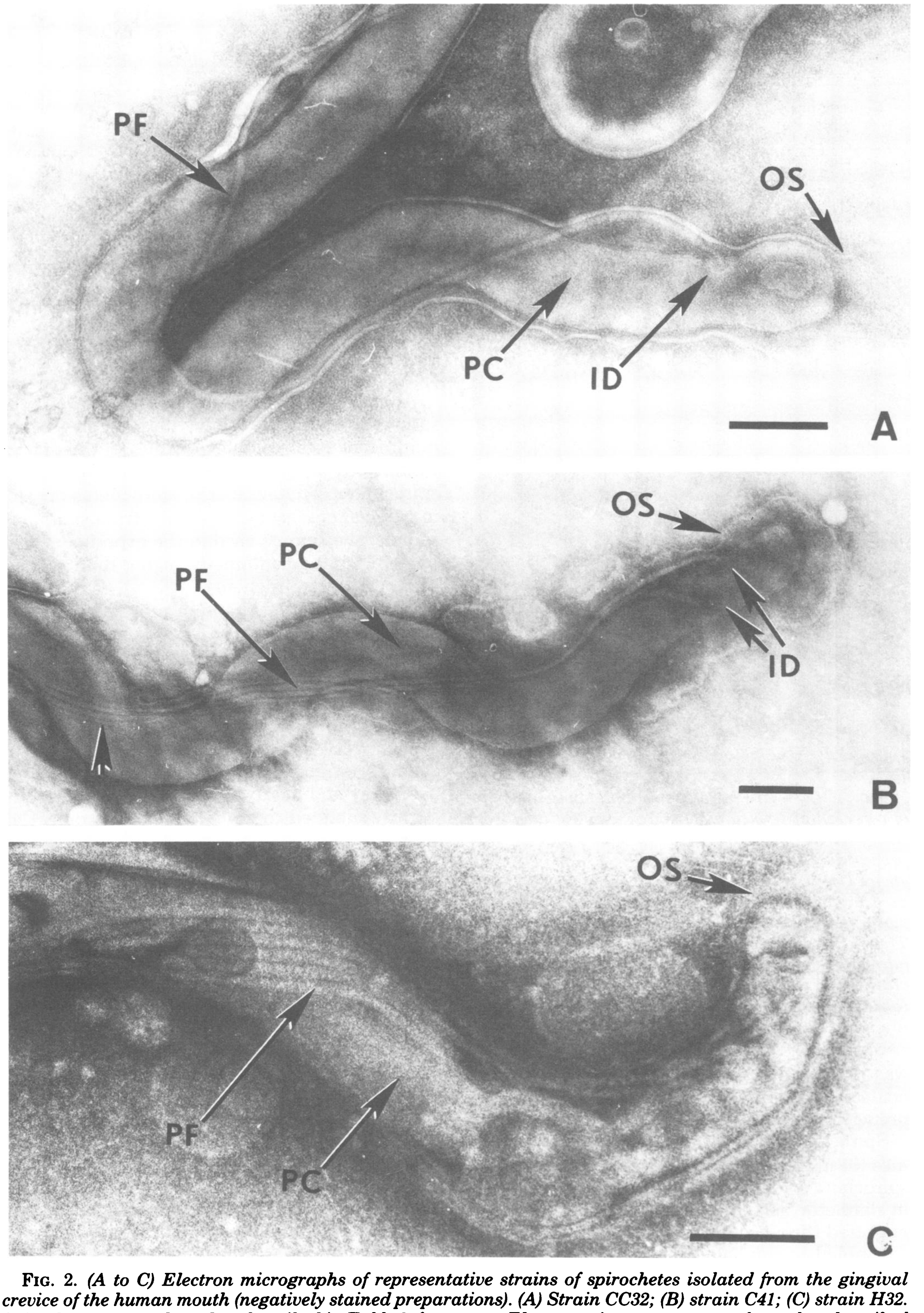

FIG. 2. *(A to C) Electron micrographs of representative strains of spirochetes isolated from the gingival crevice of the human mouth (negatively stained preparations). (A) Strain CC32; (B) strain C41; (C) strain H32. Spirochetes were cultured as described in Table 2, footnote a. Electron microscopy was performed as described previously (9). Each organism has ultrastructural features typical of spirochetes (3, 5), i.e., periplasmic fibrils (PF) wound around a protoplasmic cylinder (PC) and an outer sheath (OS). Strain CC32 (A) has one PF anchored to one end of the PC by an insertion disk (ID). In strain C41 (B), each of two PF is anchored to one end of the PC by an ID. These fibrils overlap with fibrils from the other end of the PC (small arrow). Bars = 0.2 μm.*

the more rapid growth of competing bacteria, or the media used do not support their growth. Medium GM-1 with or without added rifampin supported the growth of approximately 5% or less of the spirochetes present in gingival debris. This was determined by comparing spirochete colony counts to direct microscopic counts of spirochetes (9). Rifampin-containing media of different compositions may be useful in isolating oral spirochetes which have not yet been cultivated. For example, the procedure we describe here may prove useful for the isolation of spirochetes that participate in mixed infections of the oral cavity, e.g., in various forms of periodontal disease (6).

We have successfully used rifampin as a selective agent for the isolation of spirochetes from a number of environments (e.g., freshwater ponds, intertidal marine muds, and deep-sea water) in addition to the human gingival crevice and bovine rumen. This result, coupled with the finding that spirochetes isolated by conventional methods (without rifampin) from a variety of environments are resistant to high levels of rifampin, indicates that this is a generally applicable technique. Presently we are studying the mechanism of the natural resistance of spirochetes to rifampin. Preliminary experiments suggest that rifampin resistance may be due to a low affinity of the spirochete ribonucleic acid polymerase for the antibiotic (S. B. Leschine and E. Canale-Parola, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I20, p. 87).

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